GB 97/01153

The Patent Office

09/171671

The Patent Office
Cardiff Road
Newport
Gwent
NP9 1RH

PRIORITY DOCUMENT

RECD 26 MAY 1997
WIPO PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation and Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed

Dated 13 MAY 1997

Patents Form 1/77

atents Act 1977
(Rule 16)

The Patent Office

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form.)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

Fee: £25

1.	Your reference	37520(3)/JMD
2.	Patent application number *(The Patent Office will fill in this part)*	**9624584.0** 27 NOV 1996
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	PEPTIDE THERAPEUTICS LIMITED 321, Cambridge Science Park, Milton Road, Cambridge CB4 4WG.
	Patents ADP number *(if you know it)*	
	If the applicant is a corporate body, give the country/state of incorporation	United Kindom
4.	Title of the invention	Substrates and Inhibitors of Proteolytic Enzymes
5.	Full name, address and postcode in the United Kingdom to which all correspondence relating to this form and translation should be sent	Reddie & Grose 16 Theobalds Road LONDON WC1X 8PL
	Patents ADP number *(if you know it)*	91001

6.	If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application *(If you know it)*	Date of filing *(day month year)*

7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day month year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of

this application?
(Answer 'Yes' if:
a) any named applicant is a corporate body.
See note (d))

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document.

	Continuation sheets of this form
Description	57
Claim(s)	6
Abstract	0
Drawing(s)	16

10. If you are also filing any of the following, state how many against each item.

Priority documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	0
Request for preliminary examination and search *(Patents Form 9/77)*	0
Request for substantive examination *(Patents Form 10/77)*	0
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature: J M Davies for Reddie & Grose

Date: 26 November 199

12. Name and daytime telephone number of person to contact in the United Kingdom: J M DAVIES 0171-242 0901

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

Substrates And Inhibitors of Proteolytic Enzymes

The present invention relates to the field of compounds which are substrates or inhibitors of proteolytic enzymes and to apparatus and methods for identifying substrates or inhibitors for proteolytic enzymes.

Many therapeutically useful drugs act as enzyme inhibitors. In particular, proteolytic enzyme inhibitors have been the focus of much attention in the pharmaceutical industry, because they play a variety of roles in a multitude of biological systems. Their proteolytic activities are related to processes ranging from cell invasion associated with metastatic cancer to evasion of an immune response, as seen in certain parasitic organisms; from nutrition to intracellular signalling to the site-specific proteolysis of viral proteases and eukaryotic hormone-processing enzymes. However, the traditional random screening methods for the identification of lead molecules as inhibitors of proteolytic enzymes are often laborious and time-consuming. Therefore new and efficient methods which can accelerate the drug discovery process are greatly in demand.

We consider that proteases contain an active catalytic site which tends to become increasingly activated as the recognition pockets[1] (S_1 and S_2 etc) and (S_1' and S_2' etc) become better occupied. Therefore, it is important that

inhibitors. Therefore, we have devised a combinatorial method for the rapid identification of these binding motifs which will greatly expedite the synthesis of inhibitors of a variety of proteolytic enzymes such as aspartyl proteases, serine proteases, metallo proteases and cysteinyl proteases.

The use of a fluorescence resonance energy transfer (FRET) substrate for the analysis of proteolytic enzyme specificity was first published by Carmel.[3] Since then many different quenched fluorogenic substrates for measuring enzyme inhibition have been described in the literature.[4-11] These substrates contain a fluorophore, F, in a P position (*vide supra*), which is quenched by another group, Q, present in a P' position (*vide supra*) and separated from F by the scissile bond. The advantage of the positioning of these residues, F and Q, is that cleavage of a peptide bond occurs between the two natural residues and, therefore, represents a more natural hydrolytic event rather than the cleavage and release of a C-terminal chromophore.

For example, Bratovanova and Petkov[12] have synthesised fluorogenic substrates from peptide 4-nitroanilides. N-acylation of peptide 4-nitroanilides with the aminobenzoyl (ABz) group yielded substrates that are internally quenched by the presence of the 4-nitroanilide moiety. Upon hydrolysis of the aminoacyl-4-nitroanilide bond, the highly fluorescent N-ABz group is released attached either to an amino acid or peptide.

Immobilised libraries, where substrates are attached to a polymer or biopolymer support, have also been used for mapping protease binding sites.[13] Singh et al. reported recently that enzymatic substrate activity of 38 selected octapeptides attached *via* a linker to controlled pore glass is predictive of the same activity of similar peptides in solution. However, these results are preliminary and only for a specific example. Therefore, it is not clear whether immobilised substrates attached to polymers can reliably replace soluble substrates in mapping the hindered protease binding sites, especially since the hydrophilic or lipophilic nature of the polymer and the size of the interstices within the polymer are bound to influence the reaction between the enzyme and its substrates.

Mixtures of internally quenched, fluorogenic substrates have also recently been described in which the quencher group, Q, is 2,4-dinitrophenyl (Dnp) and is attached to the P side of the scissile bond, while the fluorogenic group, is N-methyl anthranilic acid (Nma) and is attached to the P side.[14]

The specificity of soluble peptide libraries have been determined.[15-17] Berman et al. described[18] an HPLC mass spectrometry technique in which 6 mixtures of 128 peptides were synthesised which were N-terminally labelled with the Dnp group in order to allow UV monitoring on the HPLC. The disadvantage of this approach is that each assay mixture

concentration of each separate component is limited by the size of the mixture because of overall solubility factors.

Drevin et al.[7] have suggested the use of individually synthesised fluorogenic substrates for the determination of enzyme activity using a chromophore which chelates lanthanide ions. Garmann and Phillips have suggested the use of FRET substrates in which the fluorogenic and quencher moieties are attached *via* thiol or amino functional groups after the peptide has been synthesised, but this has the disadvantage that they are not in library form and that these functional amino and thiol groups need to be selectively revealed after the peptide has been synthesised. Wang et al. have suggested the use of the EDANS and DABCYL fluorescor and quencher pairing for the individual synthesis of substrates for proteolytic enzymes.

The above methods which have used FRET techniques for the mapping of the active site around a specific protease suffer from one or more of the following disadvantages:

i. because of general aqueous insolubility they do not produce mixtures of compounds in a form suitable for high throughput screening in aqueous solution.

ii. the derivatised compounds cannot be prepared in combinatorial library form using solid phase techniques.

iii. the mixtures which have been used[8,9] were not self-decoding, and needed time-consuming deconvolutive resynthesis for identification of the active molecules.

Brief Description of the Invention

The present invention relates to the field of:

i. Compounds which are substrates or inhibitors of proteolytic enzymes.

ii. Apparatus and methods which provide the rapid generation of structure-activity relationships using auto-deconvoluting combinatorial libraries, which facilitate the invention of novel inhibitors of proteolytic enzymes.

iii. Apparatus and methods which provide the detection and measurement of proteolytic enzyme activity using combinatorial FRET (fluorescence resonance energy transfer) libraries of molecules.

iv. Apparatus and methods which provide the establishment of biological assays for proteolytic enzymes through the rapid discovery of highly active substrates for proteolytic enzymes.

We describe herein apparatus and methods which can be used for the rapid generation of structure-activity relationship (SAR) data and, therefore, the characterisation of the binding motif of any protease, and which will, therefore, facilitate:

i. the development of a sensitive enzyme inhibition

ii. the invention of novel compounds which are proteolytic enzyme inhibitors by rapid characterisation of the best binding motif.

iii. computer aided drug design to design potent inhibitors using known methodology, and also in prioritising which pre-synthesised compounds in the in-house and commercially available databases to assay.

In a first aspect the invention provides novel compounds represented by the formula A-B-C-D-nE-F [I] in which;
A represents a fluorescor internally quenched by F;
B, C, D, and E represent groups such that the scissile bond between any two of these groups is a suitable bond;
F represents a quencher capable of internally quenching the fluorescor A; and
n represents an integer between 1 and 4 inclusive.

In some embodiments the suitable bond is an unsubstituted amide bond (see Example 1); in other embodiments the suitable bond is an ester bond (see Example 2).

In a second aspect the invention provides a combinatorial library of FRET compounds comprising a mixture of compounds of formula [I].

In a third aspect the invention provides for the use of such a combinatorial FRET library in a method which provides rapid generation of structure-activity relationships (SAR) which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET

(fluorescence resonance energy transfer) molecules to find a substrate or substrates for the enzyme. According to this method an identified substrate can be synthesised and used in biological assay for proteolytic enzymes. Novel substrates are included in the scope of the invention.

In a forth aspect the invention provides for the use of such a combinatorial FRET library in a method for detection and measurement of proteolytic enzyme activity against compounds of the library.

In a fifth aspect the invention provides a method which comprises the identification of an enzyme inhibitor or inhibitors wherein a FRET compound which has been identified as a substrate is used in an inhibition assay with the enzyme separately against a panel of possible inhibitors.

In a sixth aspect the invention provides a set of compounds which comprises two complementary FRET compound libraries. Such a set will be referred to hereafter as "apparatus" because it allows for the screening or assay method for identifying substrates or inhibitors of proteolytic enzymes. This set of compounds constituting an apparatus is capable of providing an auto-deconvoluting combinatorial library as will be described below.

In a seventh aspect the invention provides a method of identifying and synthesising an inhibitor of a proteolytic

energy transfer molecules, deconvoluting the library to find a substrate or substrates for the enzyme and synthesis of an inhibitor based on the substrate or substrates. The direct product of this method is one or more novel proteolytic enzyme inhibitors.

In an eighth aspect the invention provides an inhibition assay which uses a FRET molecule, which has been identified as a substrate for the enzyme, wherein the molecule is assayed with the enzyme separately against a panel of possible inhibitors.

In a ninth aspect the invention provides a complementary pair of compound libraries L1 and L2 which constitute a set containing compounds of formula:

Aa-Bb-Cc-Dd-n(Ee)-Ff-Gg

giving a x b x c x d x e x f x g = Mn compounds in each library, there being a predetermined number (P1, P2) of mixtures each consisting of a predetermined number (Q1, Q2) of individual identifiable compounds in each library, wherein both L1 and L2 contain the same Mn compounds, but wherein any two compounds which are found together in one mixture of Q1 compounds of L1 are not found together in any one of the P2 mixtures of L2.

In a tenth aspect the invention provides a method of screening for enzymic activity using the libraries L1, L2 described above in which the P1 mixtures of L1 and the P2 mixtures of L2 are each placed separately into individual wells of two well plates, the well plates having wells

arranged in a format adapted to allow deduction of a unique active compound formula from the presence of activity in one well of the well plate of L1 and one well of the well plate of L2.

The apparatus of the invention preferably comprises two complementary compound libraries, L1 and L2, each containing n x 1600 compounds of the invention, of the type $A\text{-}B_{1\text{-}10}\text{-}C_{1\text{-}10}\text{-}D_{1\text{-}8}\text{-}n(E_{1\text{-}2})\text{-}F\text{-}G$ [II], in which:

A = a fluorescor internally quenched by F, preferably an unsubstituted or substituted anthranilic acid derivative, connected by an amide bond to B

B, C, D, E, are natural or unnatural amino acid residues connected together by suitable bonds, although B, C, D and E can be any set of groups, provided that the scissile bond between D-E is an unsubstituted bond.

F = a quencher capable of internally quenching the fluorescor A, preferably an unsubstituted or substituted 3-nitrotyrosine derivative.

G = optionally present and is a hydrophilic moiety, preferably an aspartyl amide moiety. If present, G advantageously ensures that all compounds in the library are imparted with aqueous solubility. Also, G should not be a substrate for any type of enzyme.

n = any integer between 1 and 4 inclusive.

In an alternative, the scissile bond could be between B-C or C-D.

(Note that A and F herein correspond generally and respectively to moieties F and Q of the prior art referred to above).

The numbers represented in subscript following residues B, C, D and E refer to the number of possibilities from which those residues are selected. Thus, by way of illustrative example, A-B_{1-5}-C-D-E_{1-2}-F-G represents a mixture of the following ten compounds:

A-B_1-C-D-E_1-F-G
A-B_2-C-D-E_1-F-G
A-B_3-C-D-E_1-F-G
A-B_4-C-D-E_1-F-G
A-B_5-C-D-E_1-F-G
A-B_1-C-D-E_2-F-G
A-B_2-C-D-E_2-F-G
A-B_3-C-D-E_2-F-G
A-B_4-C-D-E_2-F-G
A-B_5-C-D-E_2-F-G

The general combinatorial formula for each library can be expressed as:

$$A_1\text{-}B_{10}\text{-}C_{10}\text{-}D_8\text{-}n(E_2)\text{-}F_1\text{-}G_1 \quad [III]$$

providing 1 x 10 x 10 x 8 x n x 2 x 1x 1 = 1600n compounds.

Both compound libraries, L1 and L2, of the above type are synthesized using solid phase techniques using the Multipin approach[4] such that each library contains 1600n compounds as 80n mixtures of 20 distinct, identifiable compounds. These 20 component mixtures are then placed

separately into each of 80 wells of a 96 well plate (the other two lanes are used for control experiments) and then screened against a known quantity of the protease.

Thus it is an important part of the invention that regardless of the number of compounds contained in the two libraries L1 and L2 (1600n, where n = any integer between 1 and 4) the libraries themselves are complementary and amenable to deconvolution without recourse to resynthesis. It is also an important part of the invention that the library matrix has been especially formatted so that the most important site pairings P_2 and P_1 for proteolytic enzymes can be identified immediately without recourse to resynthesis.

Those compounds of the type A-B-C-D-E-F-G, that are the better substrates for the protease will be cleaved, and can be readily identified because the fluorescor, A, will be cleaved from its nearby quencher F, in a time dependent manner which can be easily quantified. The fluorescent quenching by F of A only occurs when the two are in nearby proximity, normally within 30 angstrom units. Hence cleavage of the bond D-E allows F to move further away from A and thus allow A to fluoresce when excited by light of the correct wavelength.

1. In this manner the most active compound can be rapidly identified without the need for further resynthesis and deconvolution. Moreover, the wells

the most efficient substrate can be found by its disappearance into its two component parts, e.g A-B-C-D and E-F-G.

Hence the problem of library deconvolution can be overcome and the most active substrate for the enzyme can be rapidly identified.

In addition, after the initial treatment of the proteolytic enzyme with the library mixtures, L1 and L2, the residual enzymatic activity in each well can be quantified by the addition of the most potent fluorogenic substrate for the enzyme, S1, which is found in the 16xn compound library. Because of the nature of the library design this can be quickly prepared and purified. If there is no appearance of increased fluorescence with the known substrate, S1, then the presence of an enzyme inhibitor can be inferred, which again can be quickly identified without the need for resynthesis.

The general description of the library layout will now be described with reference to figures 1 to 14.

For example, when n=1 and the library contains 1600 compounds, in the first column of the first row (A1) (Fig.1) in the first plate (P1) of the library L1, (hereinafter designated as location A1,P1,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_1 and E_2) (Fig. 2). In the tenth column of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component, C_{10}, one

D component, D_2, the ten B components and the two E components (E_1 and E_2). In the tenth column of the eighth row (H10) in the first plate (P1) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component, C_{10}, one D component, D_8, the ten B components and the two E components (E_1 and E_2). Hence all 1600 components are present in the one plate, because the 80 wells each contain 20 components.

A second complementary library is synthesised as follows (Fig. 3). In the first column of the first row (A1) of the first plate (P1) of the library, L2, (hereinafter designated as location A1,P1,L2), there will be ten C components, two D components (D_1 and D_2), one B component, B_1, and one E component, E_1. In the tenth column of the first row (A10) of the first plate (P1) of the library, L2, (hereinafter designated as location A10,P1,L2), there will be ten C components, two D components (D_1 and D_2), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the first plate (P1) of the library, L2, (hereinafter designated as location B1,P1,L2), there will be ten C components, two D components (D_1 and D_2), one B component, B_1, and one E component, E_2. In the tenth column of the second row (B10) of the first plate (P1) of the library, L2, (B10,P1,L2) there will be ten C components, two D components (D_1 and D_2), one B component, B_{10}, and one E component, E_2. Hence only the first two rows are used to accommodate 400 compounds in total.

location A1,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_1, and one E component, E_1 (Fig. 4). In the tenth column of the first row (A10) of the second plate (P2) of the library, L2, (hereinafter designated as location A10,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the second plate (P2) of the library, L2, (hereinafter designated as location B1,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_1, and one E component, E_2. In the tenth column of the second row (B10) of the second plate (P2) of the library, L2, (B10,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_{10}, and one E component, E_2. Hence only the first two rows are used to accommodate 400 compounds in total.

In the first column of the first row (A1) of the third plate (P3) of the library, L2, (hereinafter designated as location A1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E component, E_1 (Fig 5). In the tenth column of the first row (A10) of the third plate (P3) of the library, L2, (hereinafter designated as location A10,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the third plate (P3) of the library, L2, (hereinafter designated as location B1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E component, E_2. In the tenth column of the second row (B10)

of the third plate (P3) of the library, L2, (B10,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10}, and one E component, E_2. Hence only the first two rows are used to accommodate 400 compounds in total.

In the first column of the first row (A1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A1,P4,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E component, E_1 (Fig. 6). In the tenth column of the first row (A10) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A10,P4,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location B1,P4,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E component, E_2. In the tenth column of the second row (B10) of the fourth plate (P4) of the library, L2, (B10,P4,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10}, and one E component, E_2. Hence only the first two rows are used to accommodate 400 compounds in total.

In this fashion two complementary libraries, L1 and L2 are prepared. In library, L1, each of the 80 of wells contains a mixture of 20 components providing 1600

and furnishing the same 1600 compounds in library, L1, but in a format in which no two compounds found together in library, L1, will be found together in library, L2.

Thus it is an important part of the invention that the compounds contained in the two libraries L1 and L2 are themselves complementary, in that any two compounds which are found together in a 20 component mixture in the same location (e.g. A1P1L1) in library L1, are not found together in any of the 20 component mixtures in any location of the library L2.

In analogous examples, where separately n=2, 3 or 4, extra plates are constructed in library, L1, format to accommodate the component pairs E_3 and E_4 (n =2), E_5 and E_6 (n = 3), and E_7 and E_8 (n = 4), respectively. For the respective deconvolution libraries of the type, L2, the respective rows in the plates P1, P2, P3, and P4, are increasingly filled with the paired components D_1 and D_2, D_3 and D_4, and D_5 and D_6, and D_7 and D_8, respectively.

For example, when n = 3, and the library contains 4800 compounds, in the first column of the first row (A1) in the first plate (P1) of the library L1, (hereinafter designated as location A1,P1,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_1 and E_2). In the tenth column of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_1 and E_2). In the tenth column of the eighth row (H10) in the first

plate (P1) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component, C_{10}, one D component, D_8, the ten B components and the two E components (E_3 and E_4). Hence 1600 components are present in the one plate, because the 80 wells each contain 20 components.

In the first column of the first row (A1) in the second plate (P2) of the library L1, (hereinafter designated as location A1,P2,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_3 and E_4). In the tenth column of the first row (A10) in the second plate (P2) of the library L1, (hereinafter designated as location A10,P2,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_3 and E_4). In the tenth column of the eighth row (H10) in the second plate (P2) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component, C_{10}, one D component, D_8, the ten B components and the two E components (E_3 and E_4). Hence 1600 components are present in the one plate, because the 80 wells each contain 20 components.

In the first column of the first row (A1) in the third plate (P3) of the library L1, (hereinafter designated as location A1,P3,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_3 and E_4). In the tenth column of the first

components and the two E components (E_5 and E_6). In the tenth column of the eighth row (H10) in the third plate (P3) of the library L1, (hereinafter designated as location H10,P3,L1) there will be one C component, C_{10}, one C component, C_8, the ten B components and the two E components (E_5 and E_6). Hence 1600 components are present in the one plate, because the 80 wells each contain 20 components. In total the three plate, P1, P2 and P3, contain 1600 compounds/plate 4800 compounds in total.

For example, when n = 4, and the library contains 6400 compounds, in the first column of the first row (A1) in the first plate (P1) of the library L1, (hereinafter designated as location A1,P1,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_1 and E_2) (Fig. 7). In the tenth column of the first row (A10) in the first plate (P1) of the library L1, (hereinafter designated as location A10,P1,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_1 and E_2). In the tenth column of the eighth row (H10) in the first plate (P1) of the library L1, (hereinafter designated as location H10,P1,L1) there will be one C component, C_{10}, one D component, D_8, the ten B components and the two E components (E_1 and E_2). Hence all 1600 components are present in the one plate, because the 80 wells each contain 20 components.

In the first column of the first row (A1) in the second plate (P2) of the library L1, (hereinafter designated as location A1,P2,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E

components (E_3 and E_4) (Fig. 8). In the tenth column of the first row (A10) in the second plate (P2) of the library L1, (hereinafter designated as location A10,P2,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_3 and E_4). In the tenth column of the eighth row (H10) in the second plate (P2) of the library L1, (hereinafter designated as location H10,P2,L1) there will be one C component, C_{10}, one D component, D_8, the ten B components and the two E components (E_3 and E_4).

In the first column of the first row (A1) in the third plate (P3) of the library L1, (hereinafter designated as location A1,P3,L1) there will be one C component, C_1, one D component, D_1, the ten B components and the two E components (E_5 and E_6) (Fig. 9). In the tenth column of the first row (A10) in the third plate (P3) of the library L1, (hereinafter designated as location A10,P3,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_5 and E_6). In the tenth column of the eighth row (H10) in the third plate (P3) of the library L1, (hereinafter designated as location H10,P3,L1) there will be one C component, C_{10}, one D component, D_8, the ten B components and the two E components (E_5 and E_6).

In the first column of the first row (A1) in the fourth plate (P4) of the library L1, (hereinafter designated as location A1,P4,L1) there will be one C component, C_1, one

the library L1, (hereinafter designated as location A10,P4,L1) there will be one C component, C_{10}, one D component, D_1, the ten B components and the two E components (E_7 and E_8). In the tenth column of the eighth row (H10) in the fourth plate (P4) of the library L1, (hereinafter designated as location H10,P4,L1) there will be one C component, C_{10}, one D component, D_8, the ten B components and the two E components (E_7 and E_8).

A second complementary library is synthesised as follows. In the first column of the first row (A1) of the first plate (P1) of the library, L2, (hereinafter designated as location A1,P1,L2), there will be ten C components, two D components (D_1 and D_2), one B component, B_1, and one E component, E_1 (Fig. 11). In the tenth column of the first row (A10) of the first plate (P1) of the library, L2, (hereinafter designated as location A10,P1,L2), there will be the ten C components, two D components (D_1 and D_2), one B component, B_{10}, and one E component, E_1. In the first column of the eighth row (H1) of the first plate (P1) of the library, L2, (hereinafter designated as location H1,P1,L2), there will be the ten C components, two D components (D_1 and D_2), one B component, B_1, and one E component, E_8. In the tenth column of the eighth row (H10) of the first plate (P1) of the library, L2, (H10,P1,L2) there will be the ten C components, two D components (D_1 and D_2), one B component, B_{10}, and one E component, E_8. Hence the matrix containing all ten columns and all eight rows are used to accommodate 1600 compounds in total.

In the first column of the first row (A1) of the second plate (P2) of the library, L2, (hereinafter designated as

location A1,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_1, and one E component, E_1 (Fig. 12). In the tenth column of the first row (A10) of the second plate (P2) of the library, L2, (hereinafter designated as location A10,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the second plate (P2) of the library, L2, (hereinafter designated as location B1,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_1, and one E component, E_2. In the tenth column of the eighth row (H10) of the second plate (P2) of the library, L2, (H10,P2,L2), there will be ten C components, two D components (D_3 and D_4), one B component, B_{10}, and one E component, E_8.

In the first column of the first row (A1) of the third plate (P3) of the library, L2, (hereinafter designated as location A1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E component, E_1 (Fig. 13). In the tenth column of the first row (A10) of the third plate (P3) of the library, L2, (hereinafter designated as location A10,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the third plate (P3) of the library, L2, (hereinafter designated as location B1,P3,L2), there will be ten C components, two D components (D_5 and D_6), one B component, B_1, and one E

there will be ten C components, two D components (D_5 and D_6), one B component, B_{10}, and one E component, E_5.

In the first column of the first row (A1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A1,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B component, B_1, and one E component, E_1 (Fig.14). In the tenth column of the first row (A10) of the fourth plate (P4) of the library, L2, (hereinafter designated as location A10,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B component, B_{10}, and one E component, E_1. In the first column of the second row (B1) of the fourth plate (P4) of the library, L2, (hereinafter designated as location B1,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B component, B_1, and one E component, E_2. In the tenth column of the eighth row (H10) of the fourth plate (P4) of the library, L2, (H10,P4,L2), there will be ten C components, two D components (D_7 and D_8), one B component, B_{10}, and one E component, E_8.

The invention will now be described by reference to the following examples.

Example 1:

In this Example the proteolytic enzyme of interest is Der P1, which is found in house dust mite faeces. The example illustrates the synthesis of a number of FRET compounds in which the suitable bond is an unsubstituted amide bond, their use as a library for screening for potential

substrates of Der P1, and subsequent identification and synthesis of active inhibitors of the enzyme.

Purification of *Der pI*.

Crude mite extract (~100mg, SmithKline-Beecham, U.K) was dissolved in 5mL Phosphate Buffered Saline (PBS; 50 mM potassium phosphate; pH 7.4 containing 150 mM NaCl *Der pI* was purified by affinity column chromatography using 4C1 antibody (indoor Biotechnology, Deeside, U.K.) The crude preparation was mixed with ~2 mL of affinity resin for 2 h at 4°C and then washed with 2-3 volumes of PBS. Elution of bound protein was carried out using 5mM glycine containing 50%(v/v) ethylene glycol. Fractions(2.2 mL) were collected and neutralised with 0.8 mL of 0.2 M sodium phosphate buffer, pH 7.0. The fractions were pooled and dialysed overnight against 4 L PBS followed by a second dialysis against 2 L PBS for 2-3h. The total protein was concentrated as required by ultrafiltration (MacroSep; Flowgen, U.K.)

Synthesis of compounds

The compounds were synthesised using the Multipin approach [] using Fmoc-Rink amide Macro crowns (Chiron Mimotypes Pty., Ltd., 11 Duerdin Street, Clayton Victoria 3168, Australia) with a loading of 7 μMoles.

The amino acid residues of each of the compounds were

acids and activated pentafluorophenyl esters, in which the side-chains are protected using acid labile protecting groups known to those skilled in the art, such as Boc- (for the $-NH_2$ of Lysine, $-NH_2$ of anthranilic acid and guanidino of arginine), tBu- (for the -OH groups of serine, threonine and tyrosine), t-Bu for the -COOH group of Aspartic acid and Glutamic acid, Trityl- (for the Amide of Asparagine and Glutamine, and the amine functionality of the Histidine ring.

The N-α-fluorenylmethoxycarbonyl protecting group of the coupled residues were cleaved using 20% piperidine in dimethylformamide (DMF) for 30 minutes at 20° C. The coupling reactions for the free acids such as Boc-ABz-OH (Boc-2-aminobenzoic acid), and Fmoc-(3-nitro)tyrosine-OH were accomplished using 10 equivalents of a mixture of the free acid (1 eq.) :TBTU (0.98 eq.): HOBt (0.98 eq.) : N-methylmorpholine (1.96 eq.) in dimethylformamide (500 μL) as solvent for 5 hours at 20° C. The other amino acids were coupled as their pentafluorophenyl esters[26] for 2-6 hours.

Hence, in order to couple approximately equal ratios of each component in the mixture of the derivatised amino acids as their pentafluorophenyl esters, a solution of a total of 0.98 equivalents (relative to the amino group loading on the crown) of the mixture of amino acid pentafluorophenyl esters : HOBT (1 eq.) in DMF (500 μL) were coupled for 16 hours at 20° C. The pins were then washed well with DMF and then recoupled using the same mixture under the same conditions. A third coupling of 10 equivalents (relative to the amino group loading of the crown) for 2 hours in DMF was performed using this

coupling protocol with equimolar mixtures of the derivatised pentafluorophenyl esters of the amino acids in slightly less than 1 equivalent, it is possible to obtain approximately equal amounts of the coupled products to the crown. In this fashion the libraries are constructed with 20 compounds present on each crown. The compounds were cleaved from the crowns directly into the 80 designated wells of the desired 96 well plate. In the cleavage protocol each crown was treated with a mixture (600 μL) containing trifluoroacetic acid (95%), triethylsilane (5%) for 2 hours at 20° C. The crowns were then washed with trifluoroacetic acid (500 μL) and this was then combined with the cleavage solution.

The Fmoc-Rink amide Macro crowns (Chiron Mimotypes Pty., Ltd., 11 Duerdin Street, Clayton Victoria 3168, Australia) at 7 μMol loading per crown, were coupled with a 10 fold excess of a mixture containing L-Fmoc-Asp(O-t-Bu)-OH (1eq) using TBTU (0.98 eq) and N-methylmorpholine (1.96 eq.) in the presence of HOBt (0.98 eq.) in DMF at 0.14M concentration. After deblocking of the Fmoc group with 20% piperidine in DMF for 30 minutes and subsequent washing with DMF and then methanol, coupling of the Fmoc-(3-nitro)tyrosine-OH was accomplished using 10 equivalents of a mixture of the Fmoc-(3-nitro)tyrosine-OH (1 eq.) :TBTU (0.98 eq.): HOBt (0.98 eq.) : N-methylmorpholine (1.96 eq.) in dimethylformamide as solvent at 0.14 M concentration for 5 hours at 20° C. Removal of the Fmoc group (*vide infra*) was followed by coupling of the

In a particular example the amino acids comprising group B include Ala, Val, Leu, Ser, Asn, Gln, Glu, Lys, Phe, Pro. The amino acids comprising group C include Ala, Val, Leu, Ser, Asn, Gln, Glu, Lys, Phe, Pro. The amino acids comprising group D include Ala, Val, Ile, Leu, Nle, Ser, Glu, Phe. For n=4, the amino acids comprising group E include Ala, Val, Ile, Leu, Nle, Ser, Glu, Phe. Otherwise any selection from the amino acids can be made for n=1, 2, or 3.

The plates containing the combined cleavage solutions and were then evaporated to dryness to yield the component mixtures using a rotary centrifuge ("SPEEDVAC", Savant Instruments Inc., Farmingdale, NY) at 800 rpm for 1 hour at 20° C under a reduced pressure of 10^{-2} mmHg. Each component was then transferred to the final mother plate using a (50%: 45%: 5%) mixture of acetonitrile: water: acetic acid. The plates were then lyophilised to dryness using at 20° C under a reduced pressure of 10^{-2} mmHg, and then stored at -20° C. In this fashion libraries of the type shown in Figures 2-14 were prepared.

In further detail, the Multipin approach which was employed is described below:

Multipin Synthesis Of Potential Substrates of *Der pI*

The 'Chiron' multipin kit consists of a standard 8 x 12 pin holder containing 96 'pin stems' to which are reversibly attached 'crowns'. The 'crowns' provide a reactive polymer surface upon which a growing peptide is anchored during solid phase peptide synthesis. Each crown

(the equivalent of the peptide-resin in standard solid phase synthesis) can be considered to be an independent reactor by performing simultaneous synthesis in individual 1mL wells of industry standard 96 well plates. Each well, and thus each crown, can be charged with a unique set of reagents providing unique sequences to each crown. Common steps such as washing or removal of Nα protection can be performed concomitantly.

Synthesis is based upon the use of Nα-fluorenylmethyloxycarbonyl (Fmoc) protected amino acids. Side-chains of tri-functional amino acids are protected with acid labile groups such as trityl or tert-butyl. The addition of amino-acid residues to the growing peptide chain, a process termed 'coupling' proceeds through the utilisation of pre-formed pentafluorophenyl (pfp) esters or activation of the free acid, using the reagents HBTU or BOP in the presence of tertiary base (NMM) and HOBt as catalyst.

The experimental techniques used are fully documented (Maeji, N. J. Bray, A. M. Valerio, R. M. and Wang, W., *Peptide Research*, **8(1)**, 33-38, 1995 and Valerio, R. M. Bray, A. M. and Maeji, N. J. *Int. J. Pept. Prot. Res*, **44**, 158-165, 1994 and the main steps are briefly as follows.

General Methods

Preparation of Multipin Assembly

onto stems and slotted into the 8 x 12 stem holder in the desired pattern for synthesis.

Removal of Nα-Fmoc Protection

A 250mL solvent resistant bath is charged with 200 ml of a 20% piperidine/DMF solution. The multipin assembly is added and deprotection allowed to proceed for 30 minutes. The assembly is then removed and excess solvent removed by brief shaking. The assembly is then washed consecutively with (200mL each), DMF (5mins) and MeOH (5mins, 2mins, 2mins) and left to air dry for 15mins.

Quantitative UV Measurement of Fmoc Chromophore Release

A 1cm path length UV cell is charged with 1.2mL of a 20% piperidine/DMF solution and used to zero the absorbance of the UV spectrometer at a wavelength of 290nm. A UV standard is then prepared consisting of 5.0mg Fmoc-Asp(OBut)-Pepsyn KA (0.08mmol/g) in 3.2mL of a 20% piperidine/DMF solution. This standard gives Abs_{290} = 0.55-0.65 (at RT). An aliquot of the multipin deprotection solution is then diluted as appropriate to give a theoretical Abs_{290} = 0.6, and this value compared with the actual experimentally measured absorbance showing the efficiency of previous coupling reaction.

Coupling of amino-acid residues

Whilst the multipin assembly is drying, the appropriate Nα-Fmoc amino acid pfp esters (10 equivalents calculated from the loading of each crown) and HOBt (10 equivalents)

required for the particular round of coupling are accurately weighed into suitable containers. Alternatively, the appropriate Nα-Fmoc amino acids (10 equivalents calculated from the loading of each crown), desired coupling agent e.g. HBTU (9.9 equivalents calculated from the loading of each crown) and activation eg HOBt (9.9 equivalents calculated from the loading of each crown), NMM (19.9 equivalents calculated from the loading of each crown) are accurately weighed into suitable containers.

The protected and activated Fmoc amino acid derivatives are then dissolved in DMF (500μl for each macrocrown, e.g. for 20 macrocrowns, 20 x 10eq x 7mmoles of derivative would be dissolved in 10 000μL DMF). The appropriate derivatives are then dispensed to the appropriate wells ready for commencement of the 'coupling cycle'. As a standard, coupling reactions are allowed to proceed for 2-6 hours (depending upon nature of coupling e.g. Ala to Ala 2 hours Val to Leu 6 hours.

When coupling Fmoc amino-acid pentafluorophenyl esters, 10eq of derivative in DMF (400μl) with bromophenol blue stock solution (100μl) is used for each macrocrown. This allows monitoring of the progress of the acylation reaction through the disappearance of the deep blue coloration of bromophenol blue in the presence of unreacted amine to a pale yellow upon completion of acylation.

Bromophenol blue (20mg) is dissolved in DMF (50mL) and HOBt (10mg) added.

Washing Following Coupling

If a 20% piperidine/DMF deprotection is to immediately follow the coupling cycle, then the multipin assembly is briefly shaken to remove excess solvent washed consecutively with (200mL each), MeOH (5mins) and DMF (5mins) and deprotected (see above). If the multipin assembly is to be stored, then a full washing cycle consisting brief shaking then consecutive washes with (200mL each), DMF (5mins) and MeOH (5mins, 2mins, 2mins) is performed.

Acidolytic Mediated Cleavage of Peptide-Pin Assembly

Acid mediated cleavage protocols are strictly performed in a fume hood. A polystyrene 96 well plate (1mL / well) is labelled, then the tare weight measured to the nearest mg. Appropriate wells are then charged with a trifluoroacetic acid / triethylsilane (95:5, v/v, 600µl) cleavage solution, in a pattern corresponding to that of the multipin assembly to be cleaved.

The multipin assembly is added, the entire construct covered in tin foil and left for 2hrs. The multipin assembly in then added to another polystyrene 96 well plate (1mL /well) containing trifluoroacetic acid / triethylsilane (95:5, v/v, 600µl) (as above) for 5 mins.

The cleaved assembly is washed with DMF (200μL, 5mins), MeOH (200μL, 5mins), the spent crowns removed and discarded, the stems removed and washed by sonication in methanol (1hr, RT).

Work up of Cleaved Peptides

The primary polystyrene cleavage plate (2hr cleavage) and the secondary polystyrene plate (5min wash) (see above) are then placed in the SpeedVac and the solvents removed (minimum drying rate) for 90mins.

The contents of the secondary polystyrene plate (see above) are transferred to their corresponding wells on the primary plate using an acetonitrile / water / acetic acid (50:45:5, v/v/v) solution (3 x 150μl) and the spent secondary plate discarded.

Analysis of Products

1.0μl of each well (see above) is diluted to 400μl with 0.1%aq TFA and analysed by HPLC-MS. Column Vydac C4 (214TP52, narrowbore, 21 x 250mm). Eluents :- Solvent A = 0.1%aq trifluoroacetic acid, Solvent B = acetonitrile / 10%A. Gradient :- 10-90% B in A over 27mins, 250ml / min, 215nm UV detection. The individual substrates described below were prepared by the above methods and shown by HPLC-MS to be >95% with the correct mass.

The primary polystyrene plate (plus the washings from the secondary plate) is covered with tin foil, held to the plate with an elastic band. A pin prick is placed in the foil directly above each well and the plate placed at -80°C for 30mins. The plate is then lyophilised on the 'Heto freeze drier' overnight. Where appropriate individual peptides were then weighed and dissolved to 10mM stock solutions in DMSO prior to biological screening. Alternatively the 20 component mixture is weighed and the peptide/20 component ratio is calculated.

Further coupling of amino acid residues was carried out according to the multipin approach described above. Whilst the multipin assembly was drying, the appropriate Nα-Fmoc amino acids (10 equivalents calculated from the loading of each crown), HATU coupling agent (9.9 equivalents calculated from the loading of each crown), HOAt catalyst (9.9 equivalents calculated from the loading of each crown) and DIPEA (19.9 equivalents calculated from the loading of each crown)were accurately weighed into suitable containers.

The protected Nα-Fmoc amino acids and coupling agents were then dissolved in DMF (500 μl for each macrocrown) and activated by the addition of DIPEA. The appropriate derivatives were then dipensed to their appropriate wells and as standard coupling to each macro crown was allowed to proceed for 2 hours.

When coupling particularly hindered amino acid residues such as N-Methyl, Cα-Methyl or unusual amino acids (whose

coupling efficiency is unknown) the coupling reaction was repeated, as standard, for a further 2 hours.

Substrates for *Der pI*

Using the general techniques described above, the following compounds were prepared and assayed as potential substrates against *Der pI* purified as described above.

Peptide	Measured K_m (μM)
Abz-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	12
H-Val-Ala-Nle-Ser-TyrNO_2-Asp-NH_2	NS
H-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
Ac-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
H-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NS
H-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
Ac-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NS
Abz-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NM
Abz-Val-Ala-Nle-Ser-NH_2	NM
Abz-Val-Ala-Nle-Ser-Phe-Asp-NH_2	NM
Abz-Val-Ala-Nle-Ser-Tyr-Asp-NH_2	NM
Abz-Val-Ala-Nle-Ser-Ala-Asp-NH_2	NM
Abz-Val-Ala-Nle-Ser-Lys-Asp-NH_2	NM
Abz-Val-Ala-Nle-Ser-eAHA-Asp-NH_2	NM
Abz-Ala-Nle-Ser-Tyr(NO_2) -Asp-NH_2	NM
Abz-Nle-Ser-Tyr(NO_2) -Asp-NH_2	NS
Bz-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NM[a]
Bz(2-carboxy)-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NM[a]

Bz-Val-Ala-Nle-Ser-Tyr(NO_2)-NH_2	NM[1]
Abz-Val-Ala-Lys-Ser-Tyr(NO_2)-Asp-NH_2	14
Abz-Val-Ala-Gln-Ser-Tyr(NO_2)-Asp-NH_2	6
Abz-Val-Ala-Thr-Ser-Tyr(NO_2)-Asp-NH_2	6
Abz-Val-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	4
Abz-Val-Ala-Cha-Ser-Tyr(NO_2)-Asp-NH_2	5
Abz-Val-Ala-His-Ser-Tyr(NO_2)-Asp-NH_2	> 20
Abz-Val-Ala-ACH-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Ala-DNle-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Ala-3pyr-Ser-Tyr(NO_2)-Asp-NH_2	10
Abz-Val-Ala-Hyp-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Ala-ACP-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Lys-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	35
Abz-Val-DAla-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Tic-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-ACH-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Met(O)-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	35
Abz-Val-2Nal-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-ACP-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-DLys-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-DGln-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-3pyr-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Cha-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-DVal-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Gln-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	12
Abz-Lys-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	>15
Abz-Tic-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-ACH-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Met(O)-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	20
Abz-3pyr-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	>10
Abz-2Nal-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	15
Abz-Leu-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	18

Abz-Cha-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	9
Abz-Bip-Ala-hLeu-Ser-Tyr(NO_2)-Asp-NH_2	2.5
Abz-Bip-Ala-hLeu-Tyr-Tyr(NO_2)-Asp-NH_2	3
Abz-Bip-Ala-hLeu-Leu-Tyr(NO_2)-Asp-NH_2	3.7
Abz-Bip-Ala-hLeu-Lys-Tyr(NO_2)-Asp-NH_2	2
Abz-Bip-Ala-hLeu-Asp-Tyr(NO_2)-Asp-NH_2	5.0
Abz-Bip-Ala-hLeu-Abu-Tyr(NO_2)-Asp-NH_2	1.7
Abz-Bip-Ala-hLeu-Cha-Tyr(NO_2)-Asp-NH_2	2.5
Abz-Bip-Ala-hLeu-Met(O)-Tyr(NO_2)-Asp-NH_2	5
Abz-Bip-Ala-hLeu-Thr-Tyr(NO_2)-Asp-NH_2	2.5
Abz-Bip-Ala-hLeu-3pyr-Tyr(NO_2)-Asp-NH_2	4
Abz-Bip-Ala-hLeu-Bu^tGly-Tyr(NO_2)-Asp-NH_2	4
Abz-Bip-Ala-hLeu-Hyp-Tyr(NO_2)-Asp-NH_2	4
Abz-Phe-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-3.Pyr-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-1.Naph-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	17
Abz-2.Naph-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-Tyr-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-Bip-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	10
Abz-Lys-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	15
Abz-Glu-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	20
Abz-Leu-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NM
Abz-Hyp-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS

NS indicates that the peptide was not hydrolysed by *Der pI*.

NM indicates that the peptide was a substrate for *Der pI*, but the K_m was not measured.

NM' indicates that the peptide was a substrate for *Der pI*,

Ranked in order of cleavage rate :-Bz > n-But > Piv > Bz(2-carboxy) > Abz.

Coupling of amino acid residues was carried out according to the multipin approach described above. Whilst the multipin assembly was drying, the appropriate Nα-Fmoc amino acids (10 equivalents calculated from the loading of each crown), HATU coupling agent (9.9 equivalents calculated from the loading of each crown), HOAt catalyst (9.9 equivalents calculated from the loading of each crown) and DIPEA (19.9 equivalents calculated from the loading of each crown) were accurately weighed into suitable containers.

The protected Nα-Fmoc amino acids and coupling agents were then dissolved in DMF (500 μl for each macrocrown) and activated by the addition of DIPEA. The appropriate derivatives were then dispensed to their appropriate wells and standard coupling to each macrocrown was allowed to proceed for 2 hours.

When coupling particularly hindered amino acid residues such as N-Methyl, Cα-Methyl or unusual amino acids (whose coupling efficiency is unknown) the coupling reaction was repeated, as standard, for a further 2 hours.

The following sequences were synthesised in this way:

Peptide Sequence	Measured Km(μM)
Abz-Val-Ala-(NMe)Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-(NMe)Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS

Abz-Val-Ala-Aib-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Val-Aib-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Deg-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
nBu-D.Ser-D.Nle-D.Ala-D.Val-p.Aba-NH_2	NS
Bz-Val-Ala-Statine-Ser-eAha-NH_2	NS
Abz-p.Aba-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS
Abz-Cmpi-Nle-Ser-Tyr(NO_2)-Asp-NH_2	NS

NS-Not Substrate: Not hydrolysed by *Der pI*

NM-Substrate but not measured: Substrate for *Der pI* but not measured.

Assay procedure

Each mixture of 20 compounds in the libraries of the apparatus described herein was screened at a concentration of 1.0 μM per compound in an assay against the cysteinyl protease *Der pI*. The most active wells were identified by the rate of emission of fluorescence at 420 nm when the samples were irradiated at 320 nm. An analysis of the two complementary libraries showed that the best substrates for the enzyme were:

Abz-B-C-D-E-Tyr(NO2)-Asp-NH2

Where

B= Valine>Alanine, Glutamine, Leucine, Phenylalanine

C= Alanine>>Glutamine, or Lysine.

D= Leucine, Norleucine or Alanine>Serine

E=Serine

Abz-Val-Ala-Nle-Ser-Tyr(NO_2)-Asp-NH_2

This compound was then resynthesised as a single component using the peptide synthesis methodology described herein. The k_{cat}/K_m value for the pure substrate in the *Der pI* assay was measured as $3.5x10^4 M^{-1}s^{-1}$, and was considered to be suitably high for use in a high throughput assay for the general screening of inhibitors of *Der pI*.

High throughput assay development

Plate assays were carried out in 96 well plate format, using 0.1 ug of *Der pI* per 100 μL assay volume in each well and using 20 μM of the substrate. All assays were performed in Assay Buffer (AB; 50 mM potassium phosphate, pH 8.25 containing 1mM ethylenediaminetetraacetic acid (EDTA) and 1mM dithiothreitol (DTT). The *Der pI* enzyme is pre-activated by addition of DTT and this in incubated at room temperature for 5 min. prior to initiation of the assay. As an example for the screening methodology, each well contains a 5 μL of a 20 μM solution of the test compound in DMSO, 10 μL of a 200 μM aqueous solution of the substrate, 2, and 85 μL of *Der pI* in AB is added to initiate the reaction. Enzyme activity is monitored by fluorescence using 320 nm for excitation and 420nm for the emission wavelengths using a Labsystems Fluroskan Ascent machine. Kinetic measurements were carried out using a Hitachi F-4500 Fluorescence Spectrophotometer.

Synthesis of inhibitors of *Der pI*

The best substrate described above was shown by HPLC-mass spectroscopic analysis of the enzyme/ assay solution, to be cleaved between the Norleucine-Serine amide bond. Replacement of the terminal Abz group by a series of derivatives (e.g. Boc-, Pivaloyl, Benzoyl, and 2- carboxy-Benzoyl) affected substrate activity and specificity for the *Der pI* enzyme. With this knowledge of the P_1-P_1' cleavage site and for the P_4-P_3-P_2-P_1 motif, the compound Boc-Val-Ala-Leu-H, 4, was synthesised as shown in Scheme 1a, figure 15.

Attachment of a suitable Michael acceptor such as CH=CH-CO2Et, and -CH=CH-SO_2 Ph to the motif (Scheme 2, figure 16), provided active inhibitors of the enzyme with apparent IC_{50} values of 50nM, 1000 nM and 100nM respectively.

Preparation of an Acyloxymethylketone Series

A series of acylomethylketone compounds was prepared by the following procedures:

N-Benzoyl-L-valyl-L-alanyl-L-norleucine

N-Benzoyl-L-valyl-L-alanyl-L-norleucine was prepared by solid phase benzoylated peptide synthesis as follows:

Resin loading (step 1)

minutes. A solution of diisopropylethylamine in DCM(10ml, 57mmol in 30ml) was added over 5minutes and the resulting mixture stirred at room temperature for 2 hours. Methanol (5ml) added and reaction mixture stirred for a further 10minutes before resin filtered and washed with 3x DCM, 2x DMF, 2x 2-propanol, 2x DMF, 2x 2-propanol, methanol, 2x ether and dried under vacuum for 24 hours.

Amino acid deprotection (step 2)

Fmoc-L-norleucine loaded resin was deprotected by treatment with 20% piperidine in DMF over 4 hours. The swollen resin was filtered, washed with 5x DMF, 2x ether and dried under vacuum for 24 hours.

Peptide chain extension (step 3)

L-Norleucine loaded resin (5mmol) was added to a solution of Fmoc-L-alanine (6.23g, 20mmol), hydroxybenzotriazole (3.0g, 20mmol), 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (7.59g, 20mmol) and diisopropylethylamine (6.97ml, 40mmol) in DMF (20ml) and allowed to swell over 4 hours with mild agitation. Resin was filtered and washed with 4x DMF, 2x ether and dried under vacuum overnight.

Steps (2) and (3) were carried out repetitively with Fmoc-L-alanine and Fmoc-L-valine to afford resin bound tripeptide H-L-valyl-L-alanyl-L-norleucine.

Peptide chain benzoylation (step 4)

L-Valyl-L-alanyl-L-norleucine loaded resin (1g, approx. 1mmol) was added to a solution of benzoic acid (0.488g, 4mmol), hydroxybenzotriazole (0.6g, 4mmol), 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (1.52g, 4mmol) and diisopropylethylamine (1.40ml, 8mmol) in DMF (5ml) and allowed to swell over 6 hours with mild agitation. Resin was filtered and washed with 4x DMF, 2x ether and dried under vacuum overnight.

Resin cleavage (step 5)

N-Benzoyl-L-valyl-L-alanyl-L-norleucine loaded resin (1.0g, approx. 1mmol) was treated with a 1% solution of trifluoroacetic acid in dichloromethane (20ml) containing triethylsilane (320ml, 2mmol) for 1 hour. Resin was removed by filtration and washed with dichloromethane (3x10ml). Organic layer was collected, evaporated and titrated with ether to afford N-benzoyl-L-valyl-L-alanyl-L-norleucine (285mg). Electrospray-MS m/z 407 [MH$^+$].

Bromomethylketone formation (step 6)

N-Benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone

N-Benzoyl-L-valyl-L-alanyl-L-norleucine(140mg, 0.34mmol was suspended in dry THF(3ml) and dry DMF was added dropwise to afford homogeneity. The reaction mixture was cooled to-10°C and isobutylchloroformate (129ml, 1.0mmol) and N-methylmorpholine (109ml,1.0mmol) added with stirring

room temperature over 1 hour before a 1:1 solution of acetic acid and 50% HBr (1ml, 3.0mmol HBr) was added dropwise and stirred for 15 minutes. The organic phase was diluted with ethylacetate (40ml), washed with water (10ml), brine (10ml) and sodium bicarbonate (2x10ml), dried over $MgSO_4$, solvent removed under vacuum. This afforded an off white solid (152mg) which could be further purified as required by prep. HPLC. Electrospray-MS m/z 482 [MH^+] and 484 [MH^+].

Acyloxymethylketone formation (step 7)

N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2, 6-bis(trifluoro methyl) benzoyloxymethyl ketone

A mixture of potassium fluoride (0.1mmol, 6mg) and 2,6-bis(trifluoromethyl)benzoic acid (0.066mmol, 17mg) in dry DMF (500ml) was stirred over molecular sieves at room temperature for 5 minutes. A solution of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone (0.033mmol, 16mg) in dry DMF (500ml) was added and the reaction mixture stirred for 1 hour. The reaction mixture was passed through a short silica plug and washed with 5% methanol in dichloromethane. Solvent was removed under vacuum and the residue purified using prep. HPLC. Freeze drying afforded (6.4mg) as a white lyophilisate. Electrospray-MS m/z 660 [MH^+].

Similarly the following compounds were prepared:

N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2, 6-dimethylbenzoyloxymethyl ketone (Electrospray-MS m/z 552

[MH+]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2,6-dimethylbenzoic acid.

N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2-hydroxybenzoyloxymethyl ketone (Electrospray-MS m/z 540 [MH+]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2-hydroxybenzoic acid.

N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2,6-dichlorobenzoyloxymethyl ketone (Electrospray-MS m/z 592 [MH+] and 594 [MH+])from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2,6-dichlorobenzoic acid.

N-Benzoyl-L-valyl-L-alanyl-L-norleucine benzoyloxymethyl ketone (Electrospray-MS m/z 524 [MH+]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and benzoic acid.

N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2,3,4,5,6-pentafluorobenzoyloxymethyl ketone (Electrospray-MS m/z 614 [MH+]) from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2,3,4,5,6 pentafluorobenzoic acid.

N-Benzoyl-L-valyl-L-alanyl-L-norleucine 1,1-dimethylpropyloxymethyl ketone (Electrospray-MS m/z 504 [MH+])from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 1,1-dimethylpropanoicacid.

ketone

valyl-L-alanyl-L-norleucine bromomethyl ketone and N-benzyloxycarbonyl-D-serine-O-tert-butylether.

N-Benzoyl-L-valyl-L-alanyl-L-norleucine N(-benzyloxycarbonyl)-D-serineoxy methyl ketone (Electrospray-MS m/z 641 [MH$^+$])from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and N-benzyloxycarbonyl-D-serine.

N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2-furanoxy methyl ketone (Electrospray-MS m/z 514 [MH$^+$])from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2-furan carboxylic acid.

N-Benzoyl-L-valyl-L-alanyl-L-norleucine 2,6-dichlorophenylacyloxy methyl ketone (Electrospray-MS m/z 606 [MH$^+$], 608 [MH$^+$])from of N-benzoyl-L-valyl-L-alanyl-L-norleucine bromomethyl ketone and 2,6-dichlorophenylacetic acid.

Standard Prep. HPLC conditions were as follows: C4 preparative HPLC system (Vydac, 22x 250mm) eluting at 10ml per minute a gradient of 5-95% (90% acetonitrile (0.1% TFA)) over 30 minutes.

The following compounds were prepared by the techniques and procedures described beneath each named compound.

Preparation of Ethyl-(S)-(E)-3-((tert-butoxy carbonyl amino valyl alanyl) amino-6-methyl-hept-2-enoate

To a suspension of sodium hydride (46mg, 1.9mmol) in anhydrous THF (4ml) cooled to 0°C was added a solution of triethylphosphonoacetate (420mg, 1.9 mmol) in THF (2 ml) dropwise over 5 minutes and the mixture stirred until gas evolution ceased. The solution was added dropwise to a solution of BocVAL-CHO (600mg, 1,56mmol) in dry THF cooled to-10°C. The reaction mixture was stirred for 1 hour and saturated ammonium chloride (10ml) was added. A white solid precipitated which was removed by filtration and the filtrate was partitioned between ethyl acetate and water. The organic layer was dried with magnesium sulphate and evaporated to give an oil which was crystallised from acetonitrile water to yield the title compound, 640mg, 91%.

MS (EI +ve) required ($M^+(C_{23}H_{41}N_2O_6)+1$)=456: found($M^+$+H) =456, (($M^+$-^{t}BOC)+1) =356 (100%).

Preparation of (S)-(E)-3-((tert-butoxy carbonyl amino valyl alanyl) amino-6-methyl-hept-2-enoic acid

The ethyl ester (455mg, 1mmol) was dissolved in dioxane (10ml) and water added followed by lithium hydroxide (126mg, 3mmol). The solution was stirred for 3 hours and 1M HCl aq was added until the pH reached neutrality. The dioxane was removed by rotary evaporation and the pH adjusted to 4 with 1M HCl aq. The title compound precipitated, filtered and washed with water to yield 420mg, 98%.

Preparation of 1,1,1-Trifluoroethyl-(S)-(E)-3-((tert-butoxy carbonyl amino valyl alanyl) amino-6-methyl-hept-2-enoate

The acid (BocVAL-CO_2H) (50mg, 0.117mmol) and dimethylaminopyridine (29mg, 0.24mmol) was dissolved in dry dichloromethane (1ml) and cooled to 0°C. Water soluble carbodiimide hydrochloride salt (26mg, 0.13mmol) in 0.5ml dichloromethane was added and the solution stirred for 5 minutes. 1,1,1-Trifluoroethanol (0.017ml, 0.23mmol) in 0.5ml dichloromethane was added and the reaction was allowed to warm to room temperature after 1 hour and the reaction mixture stirred overnight. The reaction mixture was washed 2x2ml 0.5M citric acid solution, 1x2ml water, 1x2ml saturated sodium bicarbonate solution, 1x2ml water, dried with magnesium sulphate and evaporated to dryness to give the title compound.

MS (EI +ve) required (M^+($C_{23}H_{38}N_3O_6F_3$)+1)=510: found(M^++H) =510, ((M^+-^{t}BOC)+1) =410, ((M^+-^{t}Bu)+1) =454 (100%).

Preparation of Ethyl-(S)-(E)-3-(N-benzoyl valyl alanyl) amino-6-methyl-hept-2-enoate

The tert-butoxy carbonyl protected ethyl ester (16.6mg, 0.036mmol) was dissolved in 4.0M HCl in dioxane (2ml) stirred at room temperature for 30 minutes and evaporated to dryness. The residue was dissolved in DMF (0.5ml) and N-methylmorpholine (7.36mg, 0.073mmol) added followed by benzoyl chloride (5.4mg. 0.038mmol) in DMF 0.5ml. The reaction stirred for 2 hours, diluted with 0.1% trifluoroacetic acid solution (4ml) and acetonitrile (2ml)

and injected onto a C4 preparative HPLC system (22x250mm) eluting at 10ml per minute, monitoring at 215nm and a gradient of 10-90% system B over 25 minutes and holding at 90% for 15 minutes. System A = 0.1% TFA in water, system B = 90% acetonitrile, 10% system A. The peak eluting at 26-28 minutes was collected and lyophilised to a white solid, yield 4.5mg, 27%.

Analysis by MS (EI +ve) required ($M^{\cdot}(C_{25}H_3N_3O_5)+1$)=460: found($M^{\cdot}$+H) -460.

Preparation of Diethyl Phenylsulfonylmethylphosphonate

The Diethyl Phenyl sulfonylmethylphophonate was prepared using a method adapted from I. Shahak, and J. Almog. (*Synthesis*, 145,1970).

The commercially available diethyl phenylthiomethylphosphonate (1.0 ml, 4.1 mmol) was dissolved in dichloromethane (10 ml). Sulphuric acid (10 ml, 25 %) was added and the mixture cooled on ice. Solid potassium permanganate was then added in three aliquots of 0.5g with stirring. After the additions the reaction appeared to be complete. Solid sodium metabisulfite was added slowly until the mixture turned colourless. This was then extracted with ethyl acetate (x3) and the combined organic washings washed with saturated sodium bicarbonate solution followed by brine before drying over sodium sulphate. The volatiles were removed *in vacuo*. The residue

In this way the desired product, diethyl phenylsulfonylmethylphosphonate (1.0 g, quant) was obtained as a colourless solid.

The product was analysed by mass spectrometry (MS) (MALDI-TOF): required (M^+ ($C_{11}H_{15}O_5PS$) +1) = 292; obtained (M^++1) = 292

Preparation of (S)-(E)-3-((*tert*-butoxycarbonylamino-valyl)alanyl)amino-1-phenylsulfonyl-5-methyl-1-hexene

Diethyl phenylsulfonylmethylphosphonate (38 mg, 129 mmol) was dissolved in dry THF (10 ml) and then cooled to 0°C under an atmosphere of nitrogen. Sodium hydride (8 mg of 60% dispersion in oil, 200 mmol) was added and the mixture stirred for 15 mins (effervescence). The aldehyde ^{t}Boc-Val-Ala-Leu-CHO (50 mg, 129 mmol) was then added to the resulting solution and the mixture was stirred for 60 mins. The reaction was quenched by the addition of dilute hydrochloric acid (0.1 M), followed by extraction with ethyl acetate (x3). The separated organic phase was sequentially washed with saturated sodium bicarbonate solution and brine before drying over sodium sulphate. The volatiles were removed *in vacuo*. The residue was purified by flash chromatography on silica eluting with ethyl acetate/hexane 4/6. An unidentified by-product was eluted first (12 mg) followed by the desired product (S)-(E)-3-((*tert*-butoxycarbonylamino-valyl)alanyl)amino-phenylsulfonyl-5-methyl-1-hexene (22 mg, 32%) as a solid.

MS (electrospray) required (M^+($C_{26}H_{41}O_6N_3S$)+1) =523: found (M^++Na) =546, (($M-^t$Boc)+1) =424 (100%).

Preparation of Diethyl methylsulfonylmethylphosphonate

The commercially available Diethyl methylthiomethylphosphonate was converted to the title compound using the method of I. Shahak and J. Almog (*Synthesis*, 171, 1969).

Preparation of (S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-1-methylsulfonyl-5-methyl-1-hexene

Diethyl methylsulfonylmethylphosphonate (30 mg, 130 mmol) was dissolved in dry THF (5 ml) and then cooled to 0°C under an atmosphere of nitrogen. Sodium hydride (7 mg of 60% dispersion in oil, 175 mmol) was added and the mixture stirred for 15 mins (effervescence). The aldehyde tBoc-Val-Ala-Leu-CHO (50 mg, 129 mmol) was then added to the resulting solution and the mixture then stirred for 60 mins. The reaction was quenched by addition of dilute hydrochloric acid (0.1 M), followed by extraction with ethyl acetate(x3). The separated organic phase was sequentially washed with saturated sodium bicarbonate solution and brine before drying over sodium sulphate. The volatiles were then removed *in vacuo*. The residue was purified by flash chromatography on silica eluting with ethyl acetate/hexane 8/2. An unidentified by-product was eluted first (4 mg), followed by the desired product (S)-(E)-3-((tert-butoxycarbonylamino-valyl)alanyl)amino-methylsulfonyl-5-methyl-1-hexene (24 mg, 40%) as a solid.

Preparation of Ethyl diethylphosphorylmethylsulfonate

Prepared in accordance with procedure B described in L. Ghosez *et. al.* (*Tetrahedron*, 43, 5125, 1987).

The product was analysed on MS (electrospray) required $(M^+ (C_7H_{15}O_3PS)+1)=261$: Found $(M^++H)=261$, $(M^++Na)=283$.

Preparation of Diethyl(S)-(E)-3-((*tert*-butoxycarbonylamino-valyl)alanyl)amino-5-methylhexenylsulfonate.

Ethyl diethylphosphorylmethanesulfonate (36 ml, ~138mmol) was dissolved in dry THF (5 ml) and then cooled to 0°C under an atmosphere of nitrogen. Sodium hydride (8 mg of 60% dispersion in oil, 200 mmol) was added and the mixture stirred for 15 mins (effervescence). The aldehyde ^{t}Boc-Val-Ala-Leu-CHO (50 mg, 129 mmol) was added to the resulting solution and the mixture stirred for 30 mins. The reaction was quenched by addition of dilute hydrochloric acid (0.1 M), followed by extraction with ethyl acetate (x3). The separated organic phase was sequentially washed with sodium bicarbonate solution and brine before drying over sodium sulphate. The volatiles were then removed *in vacuo*. The residue was purified by flash chromatography on silica eluting with ethyl acetate/hexane 1/1. The desired product Diethyl(S)-(E)-3-((*tert*-butoxycarbonylamino-valyl)alanyl)amino-5-methylhexenylsulfonate, (22 mg, 35%) was obtained as a solid.

MS(electrospray) required(M^+($C_{32}H_{41}O_5N_3S$)+1)=492: found (M^++1)=492, ((M^+-^{t}Boc)+1)=392 (100%)

Example 2:

Design of Depsipeptides

Another suitable bond is an ester bond to form a depsipeptide. The incorporation of depsipeptide substrates aided the identification of substrates for low reactivity viral proteases.

Substrates of the general formula

$$n\ [Abz\text{-}B_{1\text{-}10}\text{-}C_{1\text{-}10}\text{-}D_{1\text{-}8}y[COO]\text{-}E_{1\text{-}8}\text{-}Tyr(NO_2)\text{-}Asp\text{-}NH_2]$$

were produced.

However, a significant proportion of viral proteases only recognise substrate sequences larger than those represented by the general structure above. It is well acknowledged that by the very nature of action of a viral protease (function is to cleave immature viral proteins into the mature viral package) one automatically receives data concerning the natural substrate sites. Thus, the general structure above can be extended by introducing extra fixed amino-acids at appropriate sites. A logical extension would be to introduce the known P1-P1' cleavage site as the depsipeptide bond, then subsequently introduce

n [Abz-B_{1-10}-C_{1-10}-D_{1-8}-E_{1-8}-P1 y[COO]-P1'-Tyr(NO_2)-Asp-NH_2]

Furthermore, if these substrates again proved to be too small, one may use the known substrate sequences to introduce additional fixed positions. For instance, with Hepatitis NS3 protease it is known that the natural P6 position is a conserved acidic residue (aspartic or glutamic acid). Thus one could extend the above structure as detailed below.

n [Abz-P6-B_{1-10}-C_{1-10}-D_{1-8}-E_{1-8}-P1 y[COO]-P1'-Tyr(NO_2)-Asp-NH_2]

The novel methodology described herein greatly facilitiates the invention of therapeutically useful proteolytic enzyme inhibitors and is commercially exploitable. This is because the best substrate motif for the proteolytic enzyme can be rapidly identified, and, since there exist in the literature a variety of ways for attaching motifs which react with the active site of a proteolytic enzyme, especially for aspartyl, metallo, serine and cysteinyl proteases, an enzyme inhibitor can be readily synthesised. Moreover, amide bond replacements or transition state mimetics can be incorporated into the molecule, which would be especially useful for the inhibition of aspartyl or metallo proteases.

The method described also facilitates the rapid development of a screening assay for novel protease

inhibitors. The most potent fluorogenic substrate discovered by library screening can subsequently be used for the detection of inhibitors of the particular proteolytic enzyme under scrutiny.

The presence of an inhibitor within the compound libraries described is readily detected by retreatment of the assay mixture with the most active fluorogenic substrate, which will allow the immediate measurement of the remaining proteolytic enzyme activity.

The invention provides self-decoding, combinatorial fluorogenic libraries, and it will greatly facilitate the design and invention of novel protease inhibitors because:

i. The peptides of the library may have increased aqueous solubility in comparison to peptides containing similar and other fluorogenic and quencher groups.

ii. The peptides are stable to contaminating exopeptidases.

iii. The self deconvolution method described, coupled with the continuous analysis of the rate of substrate cleavage data, allows the immediate identification of the most active binding motif contained within the substrate library.

iv. The method allows for the rapid assessment of the enzyme assay mixture for any compounds in the library that are acting as enzyme inhibitors.

Abbreviations

Abbreviations used herein are as follows:

Abbreviations for amino acids and nomenclature of peptide structures follow the recommendations given in: IUPAC-IUB Commission on Biochemical Nomenclature, *(J. Biol. Chem,* **247,** 997, 1971). All chiral amino acids are of the L configuration unless otherwise stated. Other abbreviations used are :

-Abu , b-amino butyric acid, : Abz, 2-amino benzoyl : ACH, 1-amino-1-carboxy-cyclohexane : ACP, 1-amino-1-carboxy-cyclopropane : Bip, Biphenylalanine : n-Bu, n-butoxycarbonyl : Bz, Benzoyl : Bz(2-carboxy), 2-carboxybenzoyl : Bu^{t}Gly, tert-Butylglycyl : BOP, benzotriazoyl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate: Cha, cyclohexylalanine : Chex, 1-carboxycyclohexyl : eAHA, gamma aminohexanoyl : HBTU, O-benzotriazoyl-N,N,N',N'-tetramethyluronium hexafluorophosphate : HOBt, 1-hydroxybenzotriazole : Hyp, trans-4-hydroxyprolinyl : hLeu, homoleucyl : 2Nal, 2-napthylalanine : NMM, N-methylmorpholine : Piv, pivoyl : 3pyr, 3-pyridylalanine : Tic, 2-carboxytetrahydroquinolyl : Tyr(NO_2), 3-nitrotyrosine.

DMF, dimethylformamide; Fmoc, fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; Pfp, pentafluorophenyl, tBoc, tert-butoxycarbonyl; tBu, tert-butyl; TFA, trifluoroacetic acid; Pmc, pentamethyl chroman, Pbf, pentamethylbenzofuran, TBTU, 2-(1H-

Benztrotriazole-1-yl)-1,1,1,3,3-tetramethyluronium tetrafluoroborate; Trt, Trityl.

p.Aba, 4-aminobenzoyl; Aib, Aminoisobutyric acid; Bip, Biphenylalanine; nBu, n-Butyl; Bz, Benzoyl; Cmpi, Carboxymethylpiperazine; Deg, Diethylglycine; DIPEA, N,N-Diisopropyl-ethylamine; HATU, O-(7-azabezotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAT, 1-hydroxy-7-azabenzotriazole; Naph, Naphthylalanine; 3.Pyr, 3-pyridiylalanine; Tyr(NO_2), 3-nitro-tyrosine.

References

1. I. Schlechter and A. Berger, Biochem. Biophys. Res. Commun., 1967, 27, 157-162)
2. A. Carmel et al., FEBS Lett., 1973, 30, 11.
3. M. M. Meldal and I. Svendsen, J. Chem. Soc. Perkin Trans.1, 1995, 1591-1596,
4. M. Meldal and K. Breddam, Anal. Biochem., 1991, 195, 141-147,
5. T. Forster, Ann. Phys., 1948, 6, 55.
6. A. Yaron, A. Carmel, and E. Katchalski-Katzir, Anal. Biochem. 1979, 95, 228 and references therein.
7. S.A. Latt et al., Anal. Biochem., 1972, 50, 56.
8. A. Persson et al., Anal. Biochem., 1977, 83, 2,96.
9. I. Yu Filppova et al., Bioorg. Khim., 1986, 12, 1172.
10. J. Pohl et al., Anal. Biochem., 1987, 165, 96.
11. S.J. Pollack et al., J. Am. Chem. Soc., 1989, 111, 5961.
12. E. K. Bratovanova and D.D. Petkov, Analytical Biochem., 1987, 162, 213.
13. J. Singh et al., J. Med. Chem., 1995, 38, 217-219 and references therein.
14. M. Green et al. in `Innovation and Perspectives in Solid Phase Synthesis' (R. Epton Ed.) Mayflower Worldwide Ltd., Birmingham, UK. 1994, 239-244.
15. J. R. Petithory et al., Proc. Natl. Acad. Sci. USA, 1991, 88, 11510-11514
16. J. Berman et al., J. Biol. Chem., 1992, 267, 1434-1437.
17. H. Drevin, A, -T. Martin, J. Carlsson, S. Oscarsson, T. Lovgren, I. Hemmila and M. Kwiatkowski, WO89/10975, (5-5-1988)

18. A. J. Garman and N. G. Phillips, WO 94/28166 (27-5-1993)

19. G.T. Wang and E.D. Matayoshi, E.P. 428000 (3-11-1989)

20. G. A. Krafft, G. T. Wang and E.D. Matayoshi, EP 428000, (3-11-1989).

21. G. R. Marshall and M. V. Toth, USP 5,164,300, (11-12-1990).

22. G. R. Marshall and M. V. Toth, USP 5,011,910, (28-12-1989).

23. K. T. Chapman, N. A. Thornberry, M. Maccoss, J. R. Weidner, R. A. Mumford, W. K. Hagmann, EP 528487A (16-8-1991)

24. R. P. Haugland, WO 93/04077, (23-8-1991)

25. R. M. Valerio, A. M. Bray N. J. Maeji, Int. J. Peptide Protein Res., 1994, 44, 158-165

26. M. Bastos, N. J. Maeji and R. H. Abeles, Proc. natl. Acad. Sci., 1995, 92, 6738-6742.

27. 'Solid Phase Peptide Synthesis', E. Atherton and R. C. Sheppard, IRL Press 1989.

Claims

1. A compound represented by the formula A-B-C-D-nE-F [I] in which;
A represents a fluorescor internally quenched by F;
B, C, D, and E represent groups such that the scissile bond between any two of these groups is a suitable bond;
F represents a quencher capable of internally quenching the fluorescor A; and
n represents an interger between 1 and 4 inclusive.

2. A compound according to claim 1 wherein A represents an unsubstituted or substituted anthranilic acid derivative.

3. A compound according to claim 1 or 2 wherein B, C, D and E independently represent natural or unnatural amino acid residues.

4. A compound according to any preceding claim wherein F represents an unsubstituted or substituted 3-nitrotyrosine derivative.

5. A compound according to any preceding claim wherein the scissile bond between D and E is an unsubstituted amide bond.

6. A compound according to any preceding claim wherein the formula further comprises G which ensures the compound is imparted with aqueous solubility.

7. A compound according to claim 6 which is represented by the formula A-B-C-D-n(E)-F-G in which

A, B, C, D, E, F and *n* are as defined in any one of claims 1 to 6; and

G represents a hydrophilic moiety which is not an enzyme substrate.

8. A compound according to claim 6 or 7 in which G represents an aspartyl amide moiety.

9. A combinatorial library of FRET compounds comprising a mixture of compounds of formula [I] of claim 1.

10. A set of compounds which comprises two complementary FRET compound libraries.

11. Apparatus which comprises an auto-deconvoluting set of compounds according to claim 10 that facilitates the invention of novel inhibitors of proteolytic enzymes and the rapid generation of structure-activity relationships (SAR) by the detection and measurement of proteolytic enzyme activity.

12. Apparatus according to claim 11 wherein the set comprises a plurality of mixtures of FRET compounds according to claim 1.

13. Apparatus according to claim 11 or 12 which comprises complementary compound libraries wherein any two FRET molecules in a single mixture of the first library are not found in a single mixture of the second library.

14. Apparatus according to any of claims 11 to 13 wherein the library comprises compounds of represented by the formula A-B_{1-3} - C_{1-3} - D_{1-3} - *n* (E_{1-3}) - F in which;
A represents a fluorescor internally quenched by F;
B,C, D and E represent groups such that the scissile bond between any two of these groups is an unsubstituted amide bond;
F represents a quencher capable of internally quenching the fluorescor A; and
n represents an integer between 1 and 4 inclusive.

15. Apparatus according to claim 14 wherein A represents an unsubstituted or substituted anthranilic acid derivative.

16. Apparatus according to claim 14 or 15 wherein B, C, D and E independently represent natural or unnatural amino acid residues.

17. Apparatus according to any one of claims 14 to 16 wherein F represents an unsubstituted or substituted 3-nitrotyrosine derivative.

18. Apparatus according to any one of claims 14 to 17 wherein the scissile bond between D and E is an unsubstituted amide bond.

19. Apparatus according to any one of claims 14 to 18 wherein the formula further comprises G which ensures that compounds in the library are imparted with aqueous solubility.

20. Apparatus according to any one of claims 14 to 19 wherein the library comprises mixtures of compounds represented by the formula A-B_{1-10} - C_{1-20} - D_{1-8} - *n* (E_{1-1}) - F-G in which

A, B, C, D, E, F and *n* are as defined in any one of claims 12 to 16 and

G represents n a hydrophilic moiety which is not an enzyme substrate.

21. Apparatus according to claim 19 or 20 in which G represents an aspartyl amide moiety.

22. Apparatus according to any one of claims 11 to 21 wherein the library comprises 1600*n* compounds as 80*n* mixtures of 20 distinct, identifiable compounds.

23. Apparatus according to claim 22 wherein the mixtures of 20 distinct, identifiable compounds are placed separately into each of 80 wells of an microtitre plate.

24. Use of a combinatorial FRET library in a method which provides rapid generation of structure-activity relationships (SAR) which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules to find a substrate or substrates for the enzyme.

25. Use of a combinatorial FRET library in a method for

26. A method of identifying and synthesising an inhibitor of a proteolytic enzyme which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules, deconvoluting the library to find a substrate or substrates for the enzyme and synthesis of an inhibitor based on the substrate or substrates.

27. An inhibition assay which uses a FRET molecule, which has been identified as a substrate for the enzyme, wherein the molecule is assayed with the enzyme separately against a panel of possible inhibitors.

28. A method which provides the rapid generation of structure-activity relationships (SAR) which comprises detection and measurement of proteolytic enzyme activity by carrying out an assay with a library of combinatorial FRET (fluorescence resonance energy transfer) molecules to find a substrate or substrates for the enzyme.

29. A method which comprises the identification of an enzyme inhibitor or inhibitors wherein a FRET compound which has been identified as a substrate is used in an inhibition assay with the enzyme separately against a panel of possible inhibitors.

30. A method according to claim 29 wherein the assay is performed by using the apparatus according to any of claims 11 to 23.

31. A method according to any proceding method claim wherein the compounds of the library are synthesized using a solid phase technique.

32. A complementary pair of compound libraries L1 and L2 which constitute a set containing compounds of formula:

Aa-Bb-Cc-Dd-n(Ee)-Ff-Gg

giving a x b x c x d x e x f x g = Mn compounds in each library, there being a predetermined number (P1, P2) of mixtures each consisting of a predetermined number (Q1, Q2) of individual identifiable compounds in each library, wherein both L1 and L2 contain the same Mn compounds, but wherein any two compounds which are found together in one mixture of Q1 compounds of L1 are not found together in any one of the P2 mixtures of L2.

33. A method of screening for enzymic activity using the libraries L1, L2 according to claim 32 in which the P1 mixtures of L1 and the P2 mixtures of L2 are each placed separately into individual wells of two well plates, the well plates having wells arranged in a format adapted to allow deduction of a unique active compound formula from the presence of activity in one well of the well plate of L1 and one well of the well plate of L2.

34. A compound, apparatus, method or assay as substantially described herein with reference to example

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10		
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10		
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10		
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10		
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10		
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10		
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10		
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10		

Figure 1

First Example of Library Matrix where n=1

Component Distribution in Plate 1, Library 1 (n=1)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D2	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D3	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D4	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D5	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D6	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D7	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D8	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}

Figure 2

Component Location in Plate 1, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}
E2	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}

Figure 3

Component Location in Plate 2, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}
E2	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}

Figure 4

Component Location in Plate 3, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E2	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}

Figure 5

Component Location in Plate 4, Library 2 (n=1).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E2	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}

Figure 6

Example Library where n=4

Component Distribution in Plate 1, Library 1 (n=4)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D2	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D3	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D4	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D5	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D6	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D7	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}
D8	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}	B_{1-10} E_{1-2}

Figure 7

Component Location in Plate 2, Library 1 n=4

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D2	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D3	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D4	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D5	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D6	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D7	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}
D8	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}	B_{1-10} E_{3-4}

Figure 8

Component Location in Plate 3, Library 1 (n=4)

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D2	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D3	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D4	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D5	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D6	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D7	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}
D8	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}	B_{1-10} E_{5-6}

Figure 9

Component Location in Plate 4, Library 1 (n=4).

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
D1	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}
D2	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}
D3	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}
D4	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}
D5	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}
D6	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}
D7	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}
D8	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}	B_{1-10} E_{7-8}

Figure 10

Component Location in Plate 1, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}
E2	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}
E3	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}
E4	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}
E5	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}
E6	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}
E7	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}
E8	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}	C_{1-10} D_{1-2}

Figure 11.

Component Location in Plate 2, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}
E2	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}
E3	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}
E4	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}
E5	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}
E6	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}
E7	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}
E8	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}	C_{1-10} D_{3-4}

Figure 12

Component Location in Plate 3, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E2	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E3	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E4	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E5	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E6	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E7	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}
E8	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}	C_{1-10} D_{5-6}

Figure 13

Component Location in Plate 4, Library 2 (n=4).

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
E1	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E2	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E3	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E4	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E5	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E6	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E7	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}
E8	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}	C_{1-10} D_{7-8}

Figure 14.

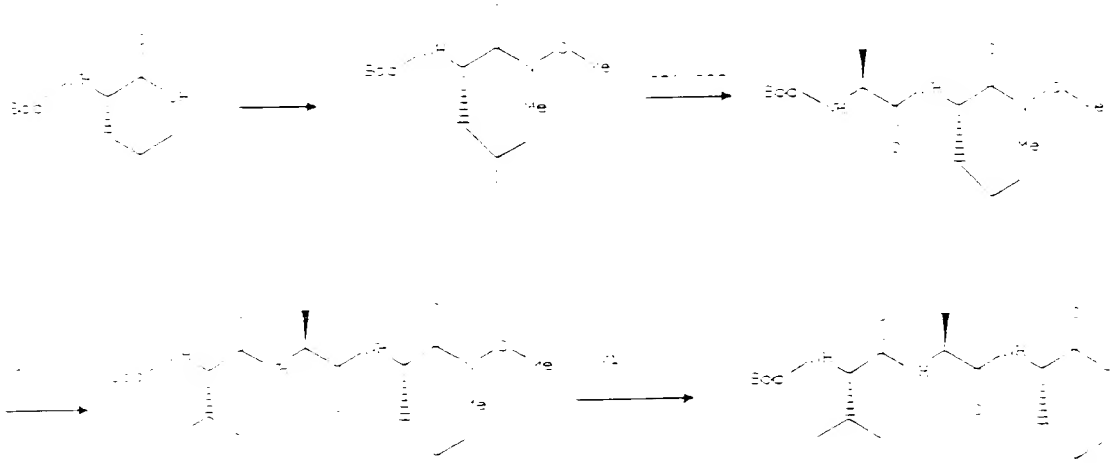

Figure 15

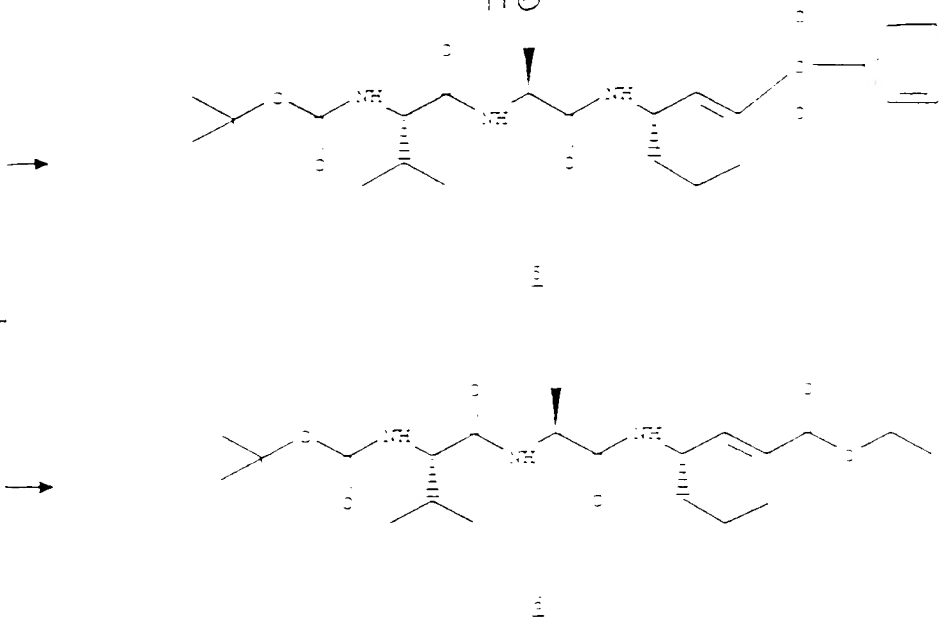

Figure 16